Exhibit B

Specific interference with gene function by double-stranded RNA in early mouse development

Florence Wianny* and Magdalena Zernicka-Goetz*†

**Wellcome/CRC Institute and Department of Genetics, University of Cambridge, Tennis Court Road, Cambridge CB2 1QR, UK*
†e-mail: mzg@mole.bio.cam.ac.uk

The use of double-stranded (ds) RNA is a powerful way of interfering with gene expression in a range of organisms, but doubts have been raised about whether it could be successful in mammals. Here, we show that dsRNA is effective as a specific inhibitor of the function of three genes in the mouse, namely maternally expressed c-*mos* in the oocyte and zygotically expressed *E-cadherin* or a *GFP* transgene in the preimplantation embryo. The phenotypes observed are the same as those reported for null mutants of the endogenous genes. These findings offer the opportunity to study development and gene regulation in normal and diseased cells.

To study early developmental events in the embryo, it is often desirable to be able to eliminate expression of a specific gene. The most valuable information would be obtained if the function of the gene of interest could be disturbed in specific cells of the embryo and at defined times. In such a situation, in the mouse, the classical techniques of gene 'knockout' cannot be used, because they eliminate gene function universally throughout the embryo. Furthermore, if a gene is repeatedly used in space and time to direct developmental processes, elimination of its role by gene 'knockout' may deny an understanding of everything but the first event. Even when the aim is to study the very first time in development at which a gene functions, the contribution of maternal transcripts and their translation products can mask the effects of the gene knockout.

There are, nevertheless, many instances in which the existing 'knockout' technology is extremely powerful. It is, however, extremely laborious. It necessitates, first, making a disrupted gene segment that is suitably marked to enable the selection of homologous recombination events in cultured embryonic stem cells. Such cells must then be incorporated into blastocysts and the resulting chimaeric animals used to establish pure breeding lines before homozygous mutants can be obtained.

Some of these difficulties could be overcome if a method for double-stranded-RNA interference (RNAi) of gene expression could be developed for mammalian cells. Such an approach, first developed in *Caenorhabditis elegans*[1], has also been shown to be

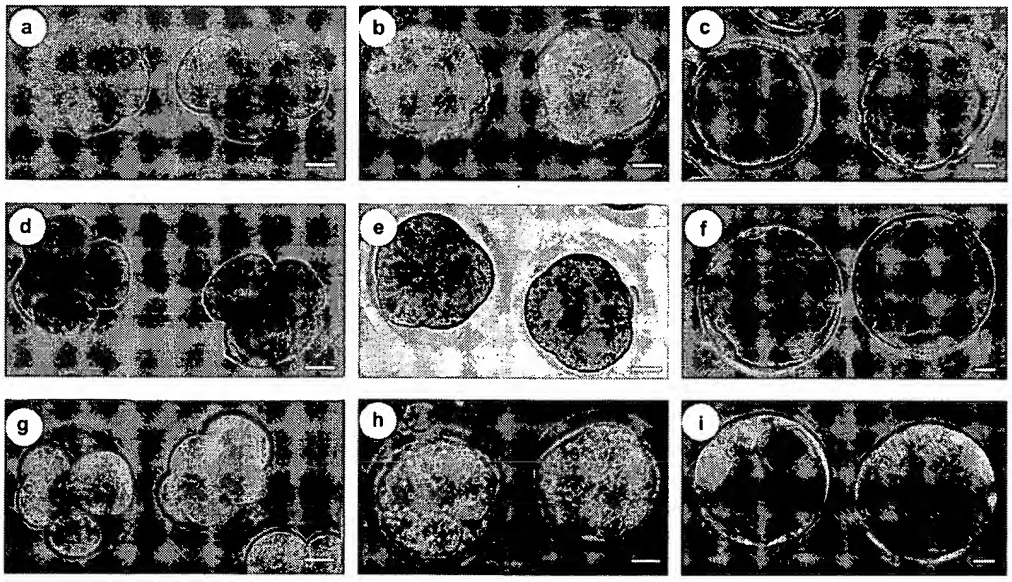

Figure 1 ***MmGFP* dsRNA specifically abrogates MmGFP expression in *MmGFP* transgenic embryos. a–c**, Representative embryos out of 131 *MmGFP* transgenic embryos obtained from 11 different matings between F_1 females and *MmGFP* transgenic males. **a**, Four- to six-cell embryos; **b**, morulae; **c**, blastocysts. A similar pattern of GFP expression was obtained after injection of antisense *MmGFP* RNA. **d–f**, Representative embryos out of 147 *MmGFP* transgenic embryos that had been injected with *MmGFP* dsRNA at the one-cell stage. **d**, Four- to six-cell embryos; **e**, morulae; **f**, blastocysts. **g–i**, Representative embryos out of 18 *MmGFP* transgenic embryos that had been injected with c-*mos* dsRNA at the one-cell stage. **g**, Six-cell-stage embryos; **h**, morulae; **i**, blastocysts. Scale bars represent 20μm.

effective in other eukaryotes, including *Drosophila melanogaster*[2], *Trypanosoma brucei*[3], planarians[4] and plants[5]. The application of this approach has also been demonstrated in zebrafish embryos, but with limited success[6]. So far there has been no report that RNAi can be used in mammals. Moreover, there are several indications of potential limitations to its function in this group of animals. Principal among these is that the accumulation of very small amounts of dsRNA in mammalian cells following viral infection results in the interferon response, which leads to an overall block to translation and the onset of apoptosis[7]. Such considerations have discouraged investigators from using RNAi in mammals.

Two factors motivated us to attempt the use of RNAi as a means of eliminating specific gene expression in the mouse embryo. First, we have developed approaches for microinjection of synthetic messenger RNAs into both mouse oocytes and preimplantation embryos as a means of successfully directing gene expression[8,9]. Second, we have established a transgenic line of mice expressing a modified form of the green fluorescent protein (MmGFP) from the ubiquitous elongation factor-1α (EF1α) promoter[10] that could provide a rapid visual assay for the effective elimination of expression of this marker gene. This facilitated assessment of whether RNAi could be effective in the mouse. Here we show that it is possible to interfere with specific gene expression in the mouse oocyte and zygote following microinjection of the appropriate dsRNA. We show that RNAi can phenocopy the effects of disrupting the maternal expression of the *c-mos* gene in the oocyte, preventing the arrest of meiosis at metaphase II. It also interferes with the zygotic expression of E-cadherin, disrupting development of the blastocyst, as also observed in the corresponding knockout mice. These studies show that RNAi can be effective in mammalian cells and this fact should have substantial implications for the analysis of gene function.

Results

dsRNA prevents expression of a *GFP* transgene. To determine whether dsRNA might be used to prevent gene expression in the mouse embryo, we developed an experimental test system using a transgenic strain of mice that expresses MmGFP under the control of the EF1α promoter[10]. This line offered the advantage that GFP expression can be easily visualized in living embryos, and, because its function is non-essential, we could monitor any non-specific deleterious effects of dsRNA on embryonic development. To avoid the complication of endurance of maternal gene products, we used heterozygous embryos in which the transgene was paternally derived. The onset of GFP expression in these embryos is seen by the appearance of green cells following the initiation of zygotic transcription at the two-cell stage.

The injection of *MmGFP* dsRNA into the single-cell zygote prevented the onset of the appearance of green fluorescence at the two- to four-cell stages. After injection, embryos were cultured *in vitro* for 3–4 days to the blastocyst stage. While uninjected embryos expressed MmGFP in the expected manner (Fig. 1a–c), all embryos injected with *MmGFP* dsRNA showed a markedly reduced green fluorescence throughout this period (Fig. 1d–f), with a minor proportion (6.8%) showing residual weak fluorescence. The embryos injected with *MmGFP* dsRNA showed normal preimplantation

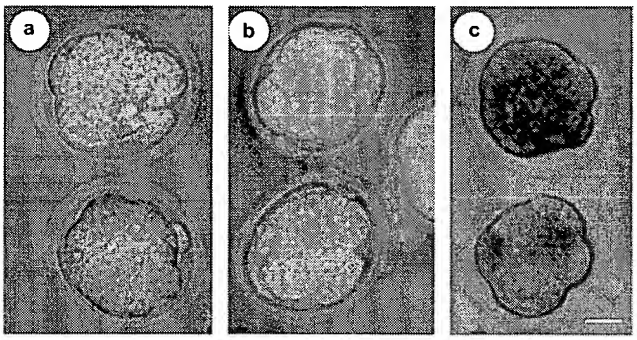

Figure 2 **Interference with expression of injected synthetic *MmGFP* mRNA.** **a–c**, Wild-type morulae injected at the one-cell stage with **a**, *MmGFP* mRNA alone; **b**, *MmGFP* mRNA together with *E-cadherin* dsRNA; **c**, *MmGFP* mRNA together with *MmGFP* dsRNA. Scale bar represents 20μm.

Table 1 Phenotypes obtained by injection of *E-cadherin* dsRNA into zygotes

Experiment	No. of embryos developing into blastocysts after injection of *MmGFP* dsRNA*	No. of embryos forming a cavity after injection of *E-cadherin* dsRNA†
1	16/21	5/21
2	7/8	9/20
3	12/24	6/60
4	10/14	5/10
5	21/22	10/19
Total	66/89 (74±5%) formed expanded blastocysts	35/130 (27±4%) formed a cavity (but did not form expanded blastocysts). The remainder failed to develop to this stage

240 uninjected zygotes were also studied. Of these, 91.6±1.1% (mean±s.e.m.) formed expanded blastocysts.

* *MmGFP* dsRNA (2 mg ml^{-1}) was injected as a control.

† 2 mg ml^{-1} *E-cadherin* dsRNA were injected.

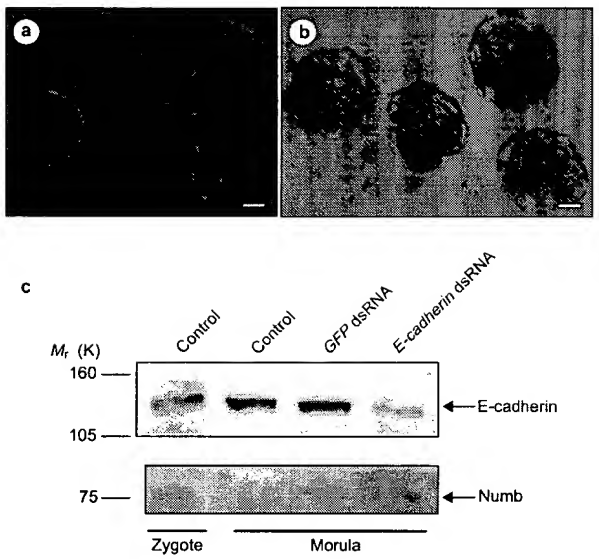

Figure 3 **Injection of *E-cadherin* dsRNA to the zygote reduces E-cadherin expression and perturbs the development of the injected embryos.** **a**, Immunofluorescent staining of E-cadherin in embryos injected at the one-cell stage with *MmGFP* dsRNA, and cultured for 4 days *in vitro* until the blastocyst stage. **b**, Immunofluorescent staining of E-cadherin in embryos injected at the one-cell stage with *E-cadherin* dsRNA, and cultured for 4 days *in vitro*. Note the altered development of these embryos. Scale bars represent 20μm. **c**, Western blot analysis of E-cadherin expression in zygotes, uninjected (control) morulae (collected at the one-cell stage and cultured *in vitro* for 3 days), morulae injected at the one-cell stage with 2 mg ml^{-1} *GFP* dsRNA and cultured *in vitro* for 3 days, and morulae injected at the one-cell stage with 2 mg ml^{-1} *E-cadherin* dsRNA and cultured *in vitro* for 3 days. The expression of Numb protein is shown as a loading control. In each case, proteins were extracted from 15 embryos. This experiment was repeated three times with the same result. The reduction of signal following injection of *E-cadherin* dsRNA was 6.5±1-fold (mean ± s.d.).

Table 2 Phenotypes observed after injection of c-*mos* dsRNA into germinal-vesicle-stage oocytes

dsRNA injected	Experiment	Number of oocytes undergoing Spontaneous activation	Fragmentation	Known null mutant phenotype
MmGFP (2 mg ml^{-1})	1	0/21	0/21	NA*
	2	0/22	2/22	
	3	1/17	0/17	
	4	0/13	0/13	
	Total	1/73 (1.4±1.4%)	2/73 (2.7±1.9%)	
c-*mos* (2 mg ml^{-1})	1	15/32	11/32	60–75% released from the metaphase-II arrest. High degree of cytoplasmic fragmentation[11,15]
	2	12/22	0/22	
	3	20/40	2/40	
	4	6/14	2/14	
	Total	53/108 (49.1±5%)	15/108 (13.9±3.3%)	
c-*mos* (0.1mg ml^{-1})	1	4/17	0/17	As above
	2	8/16	2/16	
	Total	12/33 (36 ± 8%)	2/33 (6.1 ± 4.2%)	

* NA, not applicable. Uninjected oocytes underwent spontaneous activation rarely and at a similar frequency to those injected with *MmGFP* dsRNA. Of 158 uninjected oocytes, 1.3% showed spontaneous activation and 3.8% underwent fragmentation. Totals are shown as mean percentages ± s.e.m.

development *in vitro* (Fig. 1d–f). When transferred into pseudopregnant females, they were also able to implant at the same frequency as embryos derived from uninjected zygotes (40.9% and 36.1%, respectively). We compared 18 injected embryos with 22 uninjected controls at two different postimplantation stages to determine whether they underwent normal development. Injected embryos developed into normal gastrulating embryos at 7.0 days post-coitum (d.p.c.), and were indistinguishable from control uninjected embryos (data not shown). At 8.5 d.p.c. (three- to four-somite stage) the injected embryos were also morphologically normal, showing that the injection of dsRNA is not toxic.

The interference with gene expression was specific, as shown by the fact that injection of an unrelated dsRNA, corresponding to a segment of the c-*mos* transcript, into MmGFP transgenic embryos did not result in a decrease in green fluorescence (Fig. 1g–i). The injection of c-*mos* dsRNA did not perturb the development of the embryos, consistent with the previous finding that the c-*mos* gene is not required for normal embryonic development[11]. Similarly, injection of dsRNA corresponding to a segment of the *E-cadherin* transcript into transgenic zygotes (59 embryos observed) did not result in a decrease in green fluorescence and did not shut down protein synthesis, although the phenotype of such embryos was abnormal (data not shown; see also below). Transgenic zygotes injected with antisense *MmGFP* RNA retained the green fluorescence at all preimplantation stages (37 embryos observed) (data not shown).

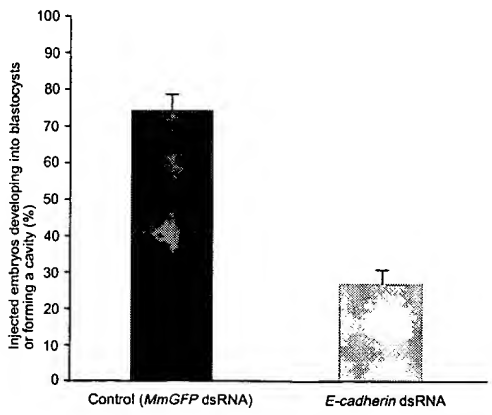

Figure 4 **Incidence of cavity formation after injection of *E-cadherin* dsRNA into the zygote.** Graphical representation of the results shown in Table 1. Dark grey, the percentage of embryos developing into blastocysts following injection of control *MmGFP* dsRNA. Light grey, the percentage of embryos forming a cavity after injection of *E-cadherin* dsRNA. Standard error bars are indicated.

We attempted to determine whether expression of MmGFP from injected capped full-length *MmGFP* mRNA could be eliminated by the co-injection of *MmGFP* dsRNA. We found that green fluorescence was greatly diminished or abolished in such injected embryos (Fig. 2c). This was in contrast to embryos injected with sense *MmGFP* mRNA, or co-injected with both sense *MmGFP* mRNA and the 'irrelevant' dsRNA for *E-cadherin* (Fig. 2a, b). Thus dsRNA can interfere with the expression of both a chromosomally located gene, and of synthetic mRNA introduced by microinjection.

Phenocopying an *E-cadherin* knockout. We then assessed the specific developmental consequences of injecting *E-cadherin* dsRNA. E-cadherin is both maternally and zygotically expressed during preimplantation development. Disruption of the *E-cadherin* gene, using homologous recombination to remove regions of the molecule esssential for adhesive function, leads to a severe preimplantation defect. These embryos can initially undergo compaction, as a result of the presence of maternally expressed E-cadherin. However, they show a defect in cavitation and never form normal blastocysts[12,13].

Following injection of *E-cadherin* dsRNA, the phenotype was identical to that of the null mutant embryos[12]. Thus, the embryos initially developed normally to the compaction stage of the morula (data not shown). However, 70% of them never formed a cavity. The remaining 30% formed a cavity, but never developed into normal blastocysts (Table 1, Figs 3b, 4). In contrast, the majority of uninjected embryos or control embryos injected with *MmGFP* dsRNA cavitated and formed normal blastocysts (Table 1, Fig. 3a).

Analysis of E-cadherin expression by immunostaining and immunoblotting showed that the expression of E-cadherin was dramatically decreased after injection of *E-cadherin* dsRNA (Fig. 3b, c). In contrast, no decrease in E-cadherin expression was observed in the embryos injected with *MmGFP* dsRNA, in which the level of E-cadherin expression was similar to that of the control uninjected embryos (Fig. 3c). The level of E-cadherin at the morula stage in embryos injected with *E-cadherin* dsRNA was lower than in newly fertilized embryos before injection (Fig. 3c). This residual E-cadherin protein may largely reflect persistence of maternally expressed protein whose synthesis ceases during the two-cell stage[14]. This residual maternal protein is present until the late blastocyst stage in

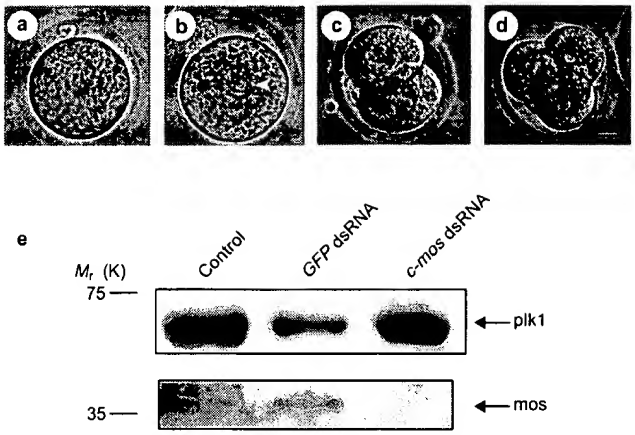

Figure 5 **Injection of c-*mos* dsRNA into immature oocytes inhibits c-mos expression and causes parthenogenetic activation. a–d**, Examples of parthenogenetically activated eggs obtained after injection of c-*mos* dsRNA in germinal-vesicle-stage oocytes. **a**, Control oocyte arrested in metaphase II; **b**, one-cell embryo (white arrow indicates the pronucleus); **c**, two-cell embryo; **d**, four-cell embryo. Scale bar represents 20 μm. **e**, Western blot analysis of c-mos expression in (left to right): oocytes arrested in metaphase II; oocytes injected at the germinal-vesicle stage with 2 mg ml^{-1} *MmGFP* dsRNA and cultured *in vitro* for 12 h; and oocytes injected at the germinal-vesicle stage with 2 mg ml^{-1} c-*mos* dsRNA and cultured *in vitro* for 12 h. In each case, proteins were extracted from 35 oocytes. The expression of polo-like kinase 1 (plk1) is shown as a loading control. This experiment was repeated three times with the same result.

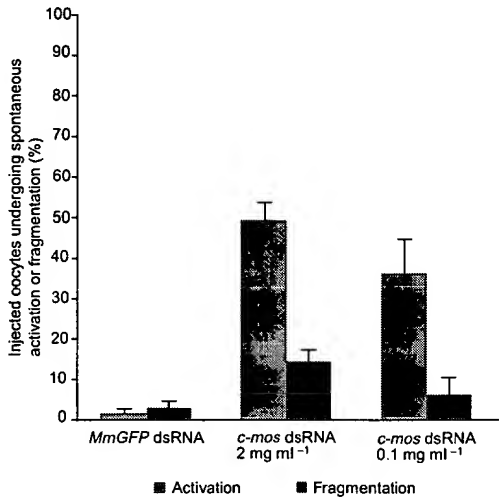

Figure 6 **Incidence of spontaneous activation and fragmentation after injection of c-*mos* dsRNA into the germinal-vesicle-stage oocyte.** Graphical representation of the results shown in Table 2. Oocytes were injected with the indicated dsRNAs. The percentage of injected oocytes undergoing spontaneous activation is shown in light grey, and the percentage undergoing fragmentation in dark grey. Standard error bars are indicated.

homozygous null embryos[12].

We conclude that injection of *E-cadherin* dsRNA leads to a striking reduction in amounts of E-cadherin protein and, consequently, a similar phenotype to that of the null mutant embryos.

dsRNA interference in the oocyte. To determine whether dsRNA might be used to interfere with maternally expressed genes, we sought a model gene producing a characteristic knockout phenotype. C-mos is an essential component of cytostatic factor, which is responsible for arresting the maturing oocyte at metaphase in the second meiotic division. In c-*mos*$^{-/-}$ mice, between 60% and 75% of oocytes do not maintain this metaphase-II arrest and instead initiate parthenogenetic development[11,15]. C-*mos* mRNA is present in fully grown immature oocytes, and its translation is initiated from maternal templates when meiosis resumes following germinal-vesicle breakdown[16]. Thus, injection of c-*mos* dsRNA would allow us to test whether dsRNA could interfere with maternal mRNA expression.

When we injected c-*mos* dsRNA into oocytes, about 63% did not maintain arrest in metaphase II (Table 2, Figs 5, 6). Of these, 78% initiated parthenogenetic development and progressed to two- to four-cell stage embryos (Fig. 5b–d). The remainder underwent fragmentation. Both of these events occur at similar frequencies in null mutant oocytes[11]. In contrast, only 1–2% of control oocytes, either uninjected or injected with *MmGFP* dsRNA, underwent spontaneous activation (Table 2, Fig. 6). 42% of injected oocytes failed to undergo metaphase-II arrest when we reduced the concentration of injected c-*mos* dsRNA by 20-fold (Table 2). We confirmed that c-*mos* dsRNA interferes with c-*mos* expression by immunoblot analysis carried out 12 h after the injection of germinal-vesicle-stage oocytes, before the phenotypic consequences of its loss of expression become apparent (Fig. 5e). Thus, injection of c-*mos* dsRNA into the oocyte specifically interferes with c-*mos* activity, mimicking the targeted deletion of c-*mos* by homologous recombination. These experiments show that dsRNA is able to block the expression of maternally provided gene products.

Discussion

We have shown that dsRNA can be used as a specific inhibitor of gene activity in the mouse oocyte and preimplantation embryo. We showed the specificity of the procedure by individually inhibiting the expression of three different genes: c-*mos* in the oocyte, and *E-cadherin* or a *GFP* transgene in the early embryo. In the cases of the two endogenous mouse genes, this results in phenotypes comparable to those of null mutants. Our experiments aimed at preventing expression of the *GFP* transgene indicate that RNAi *per se* does not affect the normal course of development.

Thus it appears that the concerns that RNAi might not work in the mouse may have been raised prematurely (reviewed in ref. 17). Concern has been expressed that the protocols used for invertebrate and plant systems are unlikely to be effective in mammals, because accumulation of dsRNA in mammalian cells can result in a general blockage of protein synthesis. The presence of extremely low concentrations of dsRNA in viral infections triggers the interferon response[18], part of which is the activation of a dsRNA-responsive protein kinase (PKR)[19]. This enzyme phosphorylates and inactivates translation factor EIF2α in response to dsRNA. The consequence is a global suppression of translation, which in turn triggers apoptosis. However, we have shown here that the injection of a dsRNA is specific to the corresponding gene; it does not cause a general translational arrest, because embryos continue to develop and we see no signs of cell death. It is possible that the early mouse embryo is incapable of an interferon response, and that there may still be difficulties in using RNAi at later stages. However, the interferon response normally occurs in response to viral infection and is usually induced experimentally using synthetic double-stranded ribonucleotide homopolymers. 'Natural' dsRNA may be less effective at inducing PKR, and the degree of induction could vary between cell types, in which case RNAi would be effective.

It has been suggested in other systems that genetic interference from injected sense or antisense RNA is actually mediated by dsRNA present at a low level in all *in vitro* RNA syntheses because of the nonspecific activity of RNA polymerases[1]. Antisense RNA has been used as a means of reducing gene expression in the embryos of a number of species. Although it has had considerable success in *Drosophila*, it has been disappointing in *Xenopus*, zebrafish and

mouse embryos. In *Xenopus*, the limitations in using the antisense approach were thought to be due to a prominent RNA-melting activity[20,21], exerted by the dsRNA-specific adenosine deaminase (dsRAD), and which itself argues against the likelihood of success for RNAi. However, although dsRAD has the potential to lead to the instability of injected dsRNA and thereby might be expected to reduce the efficacy of the approach, others have postulated that dsRNA modifed by this enzyme might actually mediate RNAi through the targeted degradation of endogenous RNA[17,22]. In the mouse embryo, the use of antisense RNA has had inconsistent and limited success in reducing gene expression, possibly because of the instability of RNA, particularly between the two–four-cell stages[23]. It has been recently reported that dsRNA might be more effective than antisense RNA in inhibiting gene expression in zebrafish embryos[6]. However, in contrast to our experiments in the mouse, these initial experiments with zebrafish embryos indicated only partial success of dsRNA interference.

Two of our experiments support the hypothesis that RNAi acts in the mouse by either inducing degradation of the targeted RNA or inhibiting its translation. First, we showed that injection of *MmGFP* dsRNA inhibits the expression of co-injected sense *MmGFP* mRNA. Second, we injected dsRNA against c-*mos* into oocytes before the germinal vesicle breaks down, the stage when c-*mos* mRNA has accumulated but has not yet been translated. C-*mos* is translated when the germinal vesicle breaks down, to arrest oocytes in metaphase of the second meiotic division[16,24]. We found that c-mos dsRNA prevents c-mos function: oocytes proceed through metaphase II and undergo parthenogenetic activation. In each case, the effects of RNAi persist for sufficient time to phenocopy the loss of gene function. As interfering with the expression of MmGFP is of no consequence to the embryo, this allows us to determine how long the RNAi effect persists. We found that although green fluorescence was absent or greatly reduced by *MmGFP* dsRNA in transgenic blastocysts injected as zygotes, fluorescence did return in embryos at 6.5 days postimplantation. During this time period, some 10–20 cells of the inner cell mass undergo a 100-fold increase in cell number, corresponding to a 40–50-fold increase in cell mass. This is coincident with a parallel increase in the expression of the transgene in uninjected embryos.

As the effects of dsRNA-mediated inhibition of gene expression persist for more than six rounds of cell division, RNAi offers new opportunities to study loss-of-function phenotypes in specific cells and at specific stages of development of the early mouse embryo. It should be possible to study the loss of function of not only any single gene, but also combinations of genes, either family members that may have redundant functions, or several members of a regulatory pathway. Moreover, the use of RNAi can be extended to evaluate the loss of function of particular genes during oocyte maturation. This also provides a means of eliminating the expression of maternally provided RNA to study maternal effects of genes that show lethality in homozygous mutants. We anticipate that it should be equally effective in other mammals, including both domestic animals and humans, in which it is difficult or impossible (because unethical) to create loss-of-function mutants and perform standard *in vivo* mutational analysis. If the approaches that we describe can be extended to the adult organism, they will have considerable therapeutic power in inhibiting gene activity in several types of disease. At the moment, in addition to allowing the analysis of genes that regulate development, elimination of gene function by RNAi in the mouse oocyte and preimplantation embryo should find widespread application in the study of genes that regulate all basic cellular processes, such as cell–cell interactions, intracellular trafficking and the cell-division cycle. □

Methods

Collection and culture of oocytes and embryos.

Immature oocytes arrested at prophase I of meiosis were collected from ovaries of 4–6-week-old F_1 (CBA × C57/Bl) mice in FHM medium (Speciality Media Inc., Lavalette, NJ) supplemented with bovine serum albumin (BSA) (4 mg ml^{-1}).

F_1 female mice were superovulated by intraperitoneal injections of pregnant mare's serum gonadotrophin (PMSG, 5 international units (i.u.)) and human chorionic gonadotrophin (hCG, 5 i. u.) 48–52 h apart. Fertilized one-cell embryos were obtained from mated females 20–24h after hCG injection.

RNA synthesis and microinjections.

The templates used for RNA synthesis were linearized plasmids. Full-length *MmGFP* complementary DNA (714 base pairs (bp)) was cloned into the T7TS plasmid. A *KpnI/HindIII* fragment of c-*mos* cDNA (550 bp) was cloned into Bluescript pSK. A cDNA fragment corresponding to exons 4–8 of *E-cadherin* (580 bp) was cloned into Bluescript pKS. RNAs were synthesized using the T3 or T7 polymerase, using the Megascripts kit (Ambion). DNA templates were removed with DNase treatment. The RNA products were extracted with phenol/chloroform, and ethanol-precipitated.

To anneal sense and antisense RNAs, equimolar quantities of sense and antisense RNAs were mixed in the annealing buffer (10 mM Tris, pH 7.4, 0.1 mM EDTA) to a final concentration of 2 μM each, heated for 10 min at 68 °C, and incubated at 37 °C for 3–4 h. To avoid the presence of contaminating single-stranded RNA in the dsRNA samples, the preparations were treated with 2 μg ml^{-1} RNase T1 (Calbiochem) and 1 μg ml^{-1} RNase A (Sigma) for 30 min at 37 °C. The dsRNAs were then treated with 140 μg ml^{-1} proteinase K (Sigma), phenol/chloroform-extracted and ethanol-precipitated. Formation of dsRNA was confirmed by migration on an agarose gel: for each dsRNA, the mobility on the gel was shifted compared to the single-stranded RNAs. For comparison of antisense and double-stranded RNAs, equal masses of RNA were injected.

RNAs were diluted in water, to a final concentration of 2–4 mg ml^{-1}. The range of effective concentrations is best illustrated by the c-*mos* RNAi experiment (Table 2) because of the sensitivity of this biological phenotype. The mRNAs were microinjected into the cytoplasm of the oocytes or embryos, using a constant flow system (Transjector, Eppendorf) as described[10]. Each oocyte or embryo was injected with ~10 pl dsRNA. Improved penetrance was achieved by using negative capacitance. After microinjection, oocytes and embryos were cultured in KSOM (Speciality Media Inc.) medium supplemented with 4 mg ml^{-1} BSA, at 37 °C in a 5% CO_2 atmosphere. *MmGFP* transgenic embryos were observed by confocal microscopy (Biorad 1024 scanning head on a Nikon Eclipse 800 microscope). Some blastocysts derived from uninjected zygotes or zygotes injected with *MmGFP* dsRNA were transferred into the uteri of pseudopregnant mothers that had been mated 2.5 days earlier with vasectomized males. Embryos were recovered either at embryonic day (E) 7.0 or E8.5, counting noon of the plug day of the pseudopregnant recipient as E0.5. They were observed by confocal microscopy as described[25].

Immunoblot and immunostaining analysis.

For immunoblot analysis, samples were subjected to SDS–PAGE and proteins were transferred to a hybond nitrocellulose membrane (Amersham). Membranes were preincubated in TBST buffer (20 mM Tris-HCl, pH 8.2, 150 mM NaCl, 0.1% Tween-20) containing 5% (*w/v*) non-fat dried milk overnight, to block nonspecific binding of antibodies. They were then incubated with the anti-E-cadherin antibody (DECMA-1), the anti-mos antibody (SantaCruz Biotechnology), the anti-Numb antibody (provided by R. Pedersen), or the anti-plk1 antibody[9] for 1 h, washed in TBST, incubated with the peroxidase-conjugated secondary antibody (SantaCruz Biotechnology) for 1 h, and washed again in TBST. The antibodies were diluted in TBST containing 5% (*w/v*) non-fat dried milk. The secondary antibody was detected by enhanced chemiluminescence (Amersham). The decrease in E-cadherin expression was quantified by comparing the optical density of the bands obtained in each western blot analysis, on a Macintosh computer using the public-domain NIH Image program.

For whole-mount immunofluorescence with anti-E-cadherin antibody, embryos were fixed in 2% paraformaldehyde for 20 min at room temperature, followed by permeabilization with 0.1% Triton X-100 for 10 min. After preincubation in 2% BSA in PBS for 30 min, embryos were incubated with the anti-E-cadherin antibody for 1 h at 37 °C, and with a Texas-red-conjugated goat anti-rat antibody (Jackson ImmunoResearch) for 1 h at 37 °C. Embryos were observed using the Biorad 1024 laser scanning confocal microscope.

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Correspondence and requests for materials should be addressed to M.Z.-G.

